Supplementary Materials and methods

P-selectin drives complement attack on endothelium during intravascular hemolysis in TLR-4/heme-dependent manner

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Methods

Reagents

The Fe^{3+} form of heme (hemin [ferriprotoporphyrin IX], designated as heme, (Sigma-Aldrich)) was dissolved to 20 mM in 50 mM NaOH and 145 mM NaCl, and further diluted in an appropriate vehicle just before use. Stock solution of PHZ of 25 mg/mL (Sigma-Aldrich) was prepared in PBS immediately before use. Inhibition of TLR4 was performed using TAK-242 molecule, diluted in PBS to a final concentration of 400 nM (Calbiochem, CAS 243984-11-4). Human plasma-derived Hx was provided by CSL Behring. To test contamination with LPS, heme solution was pre-incubated with 1µg/mL of LPS inhibitor Polymyxin B (Sigma-Aldrich, P4932).

Animal experimentation

Mouse treatment. All experiments were conducted in accordance with the recommendations for the care and use of laboratory animals and with the approval APAFIS#7135-2016100520465430v5 of the French Ministry of Agriculture. C57Bl/6 mice (WT mice) were from Charles River Laboratories, (L'Arbresle, France) and TLR4-/-(1) and C3-/-(2) were from an in house colony. Eight week old C57Bl/6, C3-/- and TLR4-/- mice were injected i.p. with 100 µL of PBS (Gibco) or with freshly prepared heme (40 µmol/kg, corresponding to 28 µg/g body weight(3)) repeated 24 hours later (day 0 and day 1). In another set of experiments WT mice were injected intravenously with 20 µmol/kg, 40 µmol/kg or 100 µmol/kg heme and sacrificed 24h later. Alternatively, mice were only injected i.p. at day 1 with PHZ (900 µmol/kg, corresponding to 0.125 mg/g body weight) and organs were recovered at day 2. In a set of experiments, mice were pretreated with i.p. injection of 40 µmol/kg of human Hx 1 h before heme or PHZ injection. Blocking antibody against P-selectin (BD Pharmingen, 553742) or its isotype control (BD Pharmingen, 5553993) was administered by retro-orbital injection 5 min

before injection of heme, PHZ or vehicle. Concentrations and route of administration were chosen as described(4, 5). At the endpoint, organs were recovered and frozen in nitric liquid and stock to -80°C. We used male and female C57Bl6/J and TLR4-/- or C3-/- mice; sex-matched animals were used for each experiment.

WT mice were pre-treated with blocking antibody against P-selectin or irrelevant IgG by i.v. injection in the eye 15 min prior i.p. injection of PBS, free heme or PHZ. In another set of experiments, WT mice were injected i.p. with isotype control or the anti-C5 blocking antibody BB5.1 (Hycult, HM1073-10B) at 1mg/mouse. Two hours later PHZ was administrated i.p. as above and mice were sacrificed after 24h.

mRNA level analyses

Snap-frozen liver sections were recovered in RLT buffer (Qiagen)+ 1% β-mercaptoethanol (Gibco) and used for mRNA extraction and gene expression analyzed by RTqPCR as described(4) previously for kidney sections. NGAL expression was normalized on actin housekeeping gene expression.

Immunofluorescence on mouse tissue.

Five µm thick frozen sections of mouse livers were cut with Cryostat Leica AS-LMD and fixed in acetone on ice for 10 min. Complement deposition was studied using rat anti-mouse C3 fragments (C3bi/C3b/C3c) (Hycult, HM1078), visualized with goat anti-rat AF555 (Thermoscientific, A-21434). NGAL expression was studied using goat anti-mouse NGAL (R&D system, AF1857), revealed by donkey anti-goat AF555 or AF647 (Thermoscientific, A21432 and A21447). Endothelial cells were visualized with two EC markers, vWF using polyclonal sheep anti-human vWF (Abcam, ab11713) revealed by donkey anti-sheep AF647 (Thermoscientific, A21448), and CD31 using polyclonal rabbit anti-mouse CD31 (Abcam,

ab124432) revealed by goat anti-rabbit AF647 (Thermoscientific, A21245). Granulocytes and macrophages were respectively labeled with rat monoclonal anti-mouse Ly6G-PE (BD Pharmingen™, 551461) and rat monoclonal anti-mouse CD68-PE (eBioscience™, 12-0681-80, FA-11). Epithelial cells were marked by monoclonal rabbit anti-mouse cytokeratin 7 (Abcam, ab181598), revealed by goat anti-rabbit (Thermoscientific, A21245), and platelets, with monoclonal rat anti-mouse CD41a-APC (eBioscience™, 17-0411-82 MWReg30). Stained slides were scanned by slide scanner Axio Scan (Zeiss). Quantification of the staining was performed using Visiopharm software (Visiopharm A/S, Hørsholm, Denmark). The colocalization of vWF and the C3 activation fragments was used to quantify the complement deposits on vascular endothelium in the liver as % of double positive area. The results were presented as fold change (FC), compared to the results of the PBS-injected mice. This organ has high amount of easily identifiable macro-vessels to allow robust analyses of macro and microvasculature. The NGAL staining is quantified as % of positive area and presented as FC compared to the results of the PBS-injected mice

ALT activity quantification. ALT activity in mouse plasma was quantified using a colorimetric assay (Sigma, MAK052-1KT), expressed in nmol/min/µL following the protocol provided by the manufacturer.

Detection of C5a in mouse plasma. C5a levels in mouse plasma was quantified using a ELISA assay, using Purified Rat Anti-Mouse C5a (BD Pharmingen, Clone I52-1486) as capture and Biotin Rat Anti-Mouse C5a (BD Pharmingen, Clone I52-278) as detection antibody. The levels of C5a were compared for every mice at basal level (day –3) and 24h after injection of PHZ. The prevention of the PHZ-mediated increase of C5a in the plasma after injection of the anti-C5 blocking antibody Bb5.1 was used as marker to validate the antibody efficacy.

Complement activation and complement receptors on EC

Flow cytometry. HUVEC were cultured in 24-wells plates as previously described(6, 7), exposed to heme at the indicated doses for 30 min at 37 °C in serum-free M199 cell culture medium (Gibco) in presence or absence of TAK-242 (Calbiochem, 614316). For evaluation of complement activation, HUVEC either washed and further exposed to NHS diluted to 33% in M199 medium for 30 min at 37 °C. HUVEC were used for experiments until passage 4. Cells were washed, detached, labeled and fixed in 0.5% formaldehyde. For studying non-covalent binding of complement deposition, cells were briefly washed 3 times with either normal M199 2% FCS or with M199 2% FCS adjusted to pH 2.5 (acidic wash). Cells were analyzed by flow cytometry (BD LSR II), and further analyzed by FlowJo X. The following antibodies were used for staining: anti-human MCP-RPE (BIORAD, MCA2113PE), anti-human C3aR-PE (BioLegend, 345804), anti-human C5aR-APC (BioLegend, 344310), in addition to the corresponding isotype controls. After incubation with serum, staining was performed with anti-properdin mouse monoclonal IgG1 (Quidel, A233), anti-FH mouse monoclonal IgG1 (Quidel, A254), anti-C3c mouse monoclonal IgG1 antibody (Quidel, A205), anti-C3a rabbit polyclonal antibody (CompTech, A218), or with anti-C5b-9 mouse monoclonal IgG1 (kindly provided by Prof. Paul Morgan, Cardiff University) or an isotype control and revealed by anti-mouse-IgG1-PE secondary antibody (Beckman Coulter, IM0551) or goat anti-rabbit IgG-AF555 secondary antibody (ThermoFisher, A21429). Blocking antibody against P-selectin (R&D systems, AF137) was diluted in M199 FCS-free medium supplemented with 10mM EGTA and 2mM $MgCl_2$ to a final concentration of 25 µg/ml or with a polyclonal sheep antibody as an irrelevant antibody (R&D systems, 5-001). HUVEC were stained for C3 deposition and C5b-9 formation as described below.

Gene silencing. At 80% confluency, HUVEC have been cultured in F12K medium, supplemented with 2 mM L-glutamine, 0.1 mg/mL heparin, 0.05 mg/mL ECGS (Merck, 02-102) and 10% FCS. HUVEC were transfected in Opti-MEM medium (Gibco) supplemented with lipotransfectamine (Thermo Fisher Scientific) and 4 nM of MCP siRNA (Qiagen, SI02654883) for 20 min at room temperature.

Immunofluoresence. HUVEC were cultured on slides in 24-well plates. Cells were fixed in paraformaldehyde 4% after staining of P-selectin, CD31, vWF, p38, p65. Respectively, staining was performed with anti-human P-selectin primary antibody (BIORAD, MCA796), rabbit anti-human vWF (DAKO, A0082), mouse anti-human CD31 (Ancell, 180-020), phospho-p38 (Thr180/Tyr182) (#9211, Cell Signaling), phospho-p65 (Ser536) (#3033, Cell Signaling) or matched isotype controls. Markers were revealed with rabbit anti-mouse AF-488 secondary antibody (ThermoFisher, A21204) or goat anti-rabbit AF555 (ThermoFisher, A21429) and DAPI (to stain nuclei). Staining was analyzed by Axiovert 200M microscope (Zeiss).

Cellular ELISA. HUVEC cells were cultured on slides in 24-well plates. HUVEC were exposed to 200 ng/mL LPS for 1 h. Cells were washed and stained for vWF HRP (DAKO, P022602) or P-selectin primary antibody (BIORAD, MCA796) and revealed with secondary anti-mouse HRP antibody (Santa Cruz, sc-2005).

Western Blot. Cells were treated with 25 µM of heme from 30 min to overnight, 100 ng/mL of LPS as positive controls. Cells were washed, detached and incubated 30 min at 4°C in RIPA lysis buffer. Phosphorylation of p65 was confirmed by WB using Phospho-65 (Ser536) (#3033, Cell Signaling), controlled with total NF-κB p65 (D14E12) (#8242, Cell Signaling) using the

SNAP technology (Merk Millipore), followed by anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling). The signal was revealed by chemiluminescence.

Surface Plasmon Resonance. Binding studies were performed using the ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories), based on SPR technology. Recombinant human P-selectin (R&D Systems) was covalently coupled via amine coupling in 50 mM sodium acetate at pH 4,8 to a sensor chip. The immobilization of concentration of P-selectin resulted in 1,600 resonance units, corresponding to a surface density of 1,3 ng/mm^2. C3(H2O) was generated by freezing/thawing native C3. For this reason, the exact quantity of C3(H2O) could not be determined, thus preventing calculation of binding affinity. P-selectin–coated channel was corrected for non-specific binding to the control channel (no protein; between 5 and 10%). P-Selectin binding at equilibrium was determined at increased concentrations of C3b (A112, CompTech), and freeze/thaw C3 (A113, CompTech) (0.0625, 0.125, 0.25, 0.5 and 1 µM). The set of sensorgrams were best fit to the 1:1 Langmuir binding model using the BIA evaluation software provided by the manufacturer.

Complement activation in serum. Heme or LPS were diluted to final concentration of 0, 2 and 100 µM in 33% NHS and incubated for 30 mins at 37°C. Bb and sC5b-9 levels in the serum were evaluated by ELISA kit following the instructions of the manufacturer (MicroVue Bb Plus EIA and MicroVue SC5b-9 Plus EIA, Quidel).

Statistics

Results were analyzed using a statistical software package (GraphPad Prism 5) as indicated in the figures legends.

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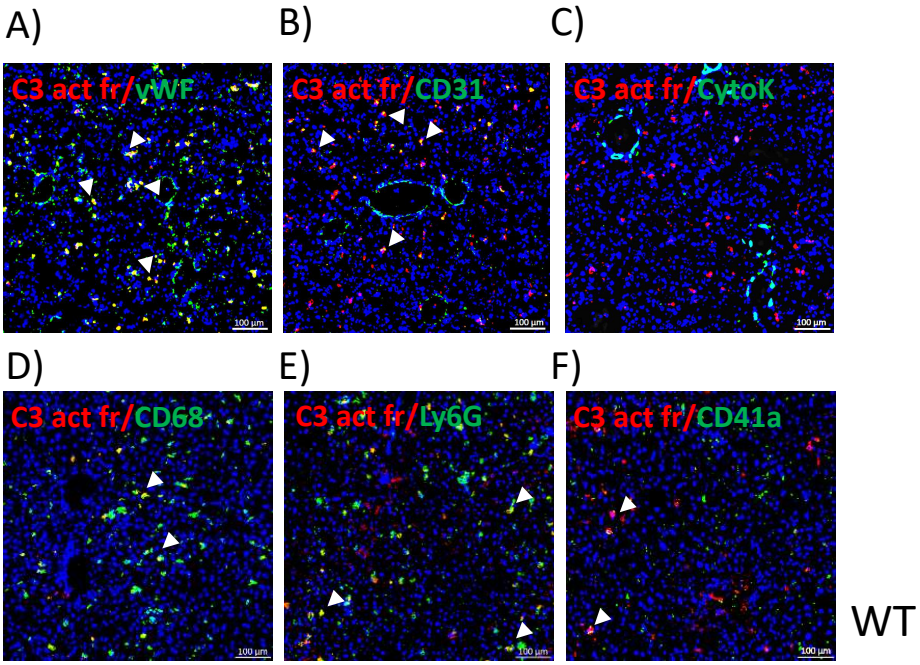
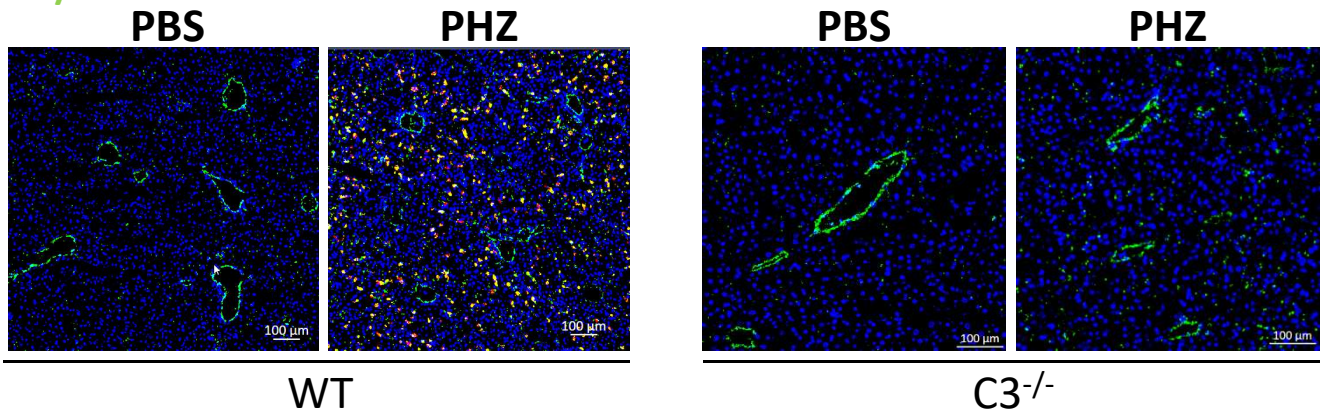
Figure S1



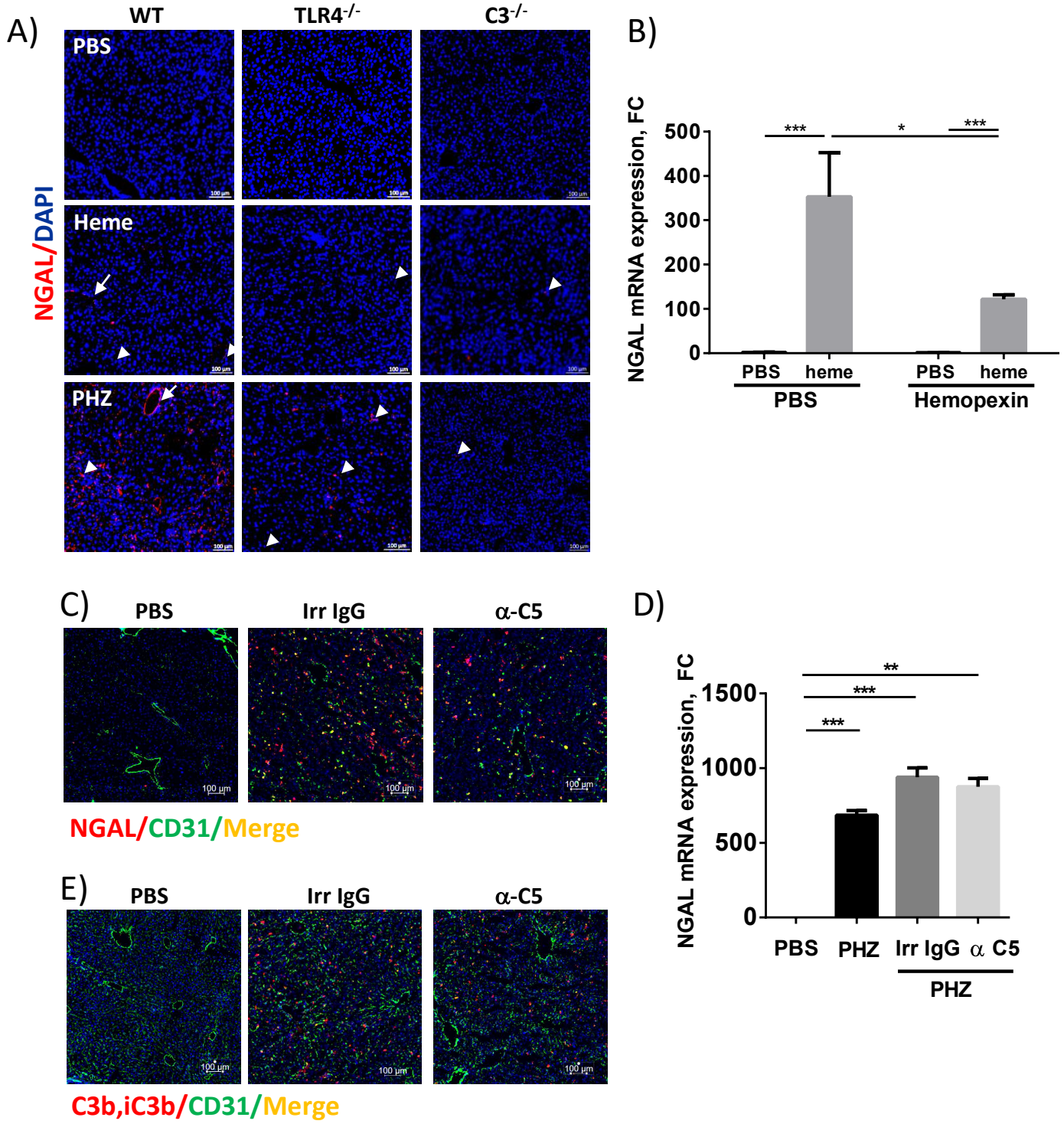
Supplementary figure S1: A-C) C3 activation fragments deposition on sinusoids' endothelium in the liver parenchyma. Double staining for C3 activation fragments (red) and respectively in green (A) vWF, (B) CD31 and (C) cytokeratin 7, of WT mice liver frozen sections treated with PHZ. White arrowheads point to co-localizations (orange). D-F) Cells infiltration and C3 activation fragments (C3 act fr) deposition in the liver parenchyma. Double staining for C3 activation fragments (red) and respectively in green (D) CD68, (E) Ly6G and (F) CD41a of WT mice liver frozen sections treated with PHZ. White arrowheads point to co-localizations (orange).

Figure S2



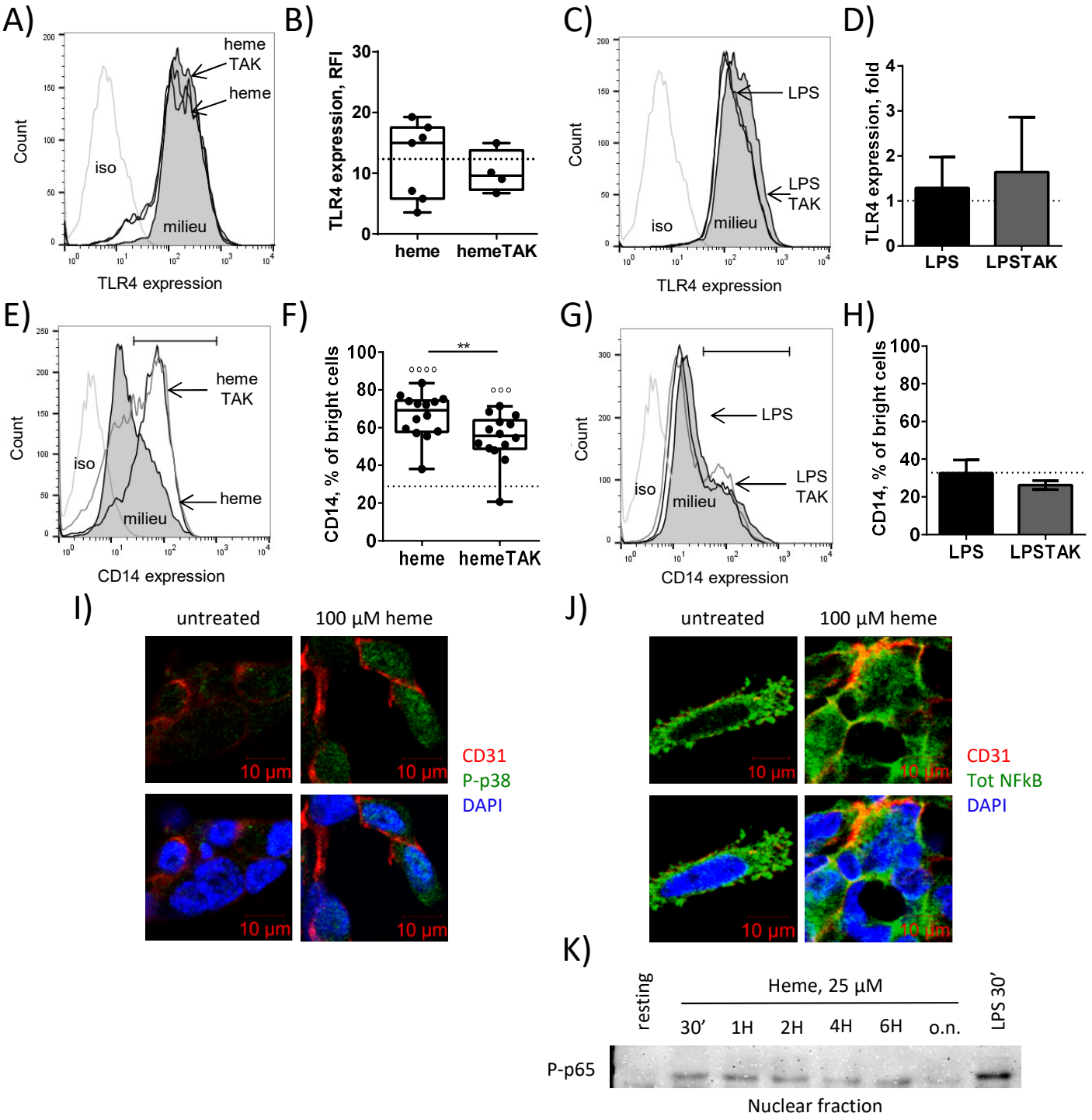
Supplementary figure S2: Staining for C3 activation fragments (C3b/iC3b, red) and vWF (green) on 5µm thick sections of frozen liver of mice injected with PBS or PHZ treated-mice in WT and $C3^{-/-}$ genetic background.

Figure S3

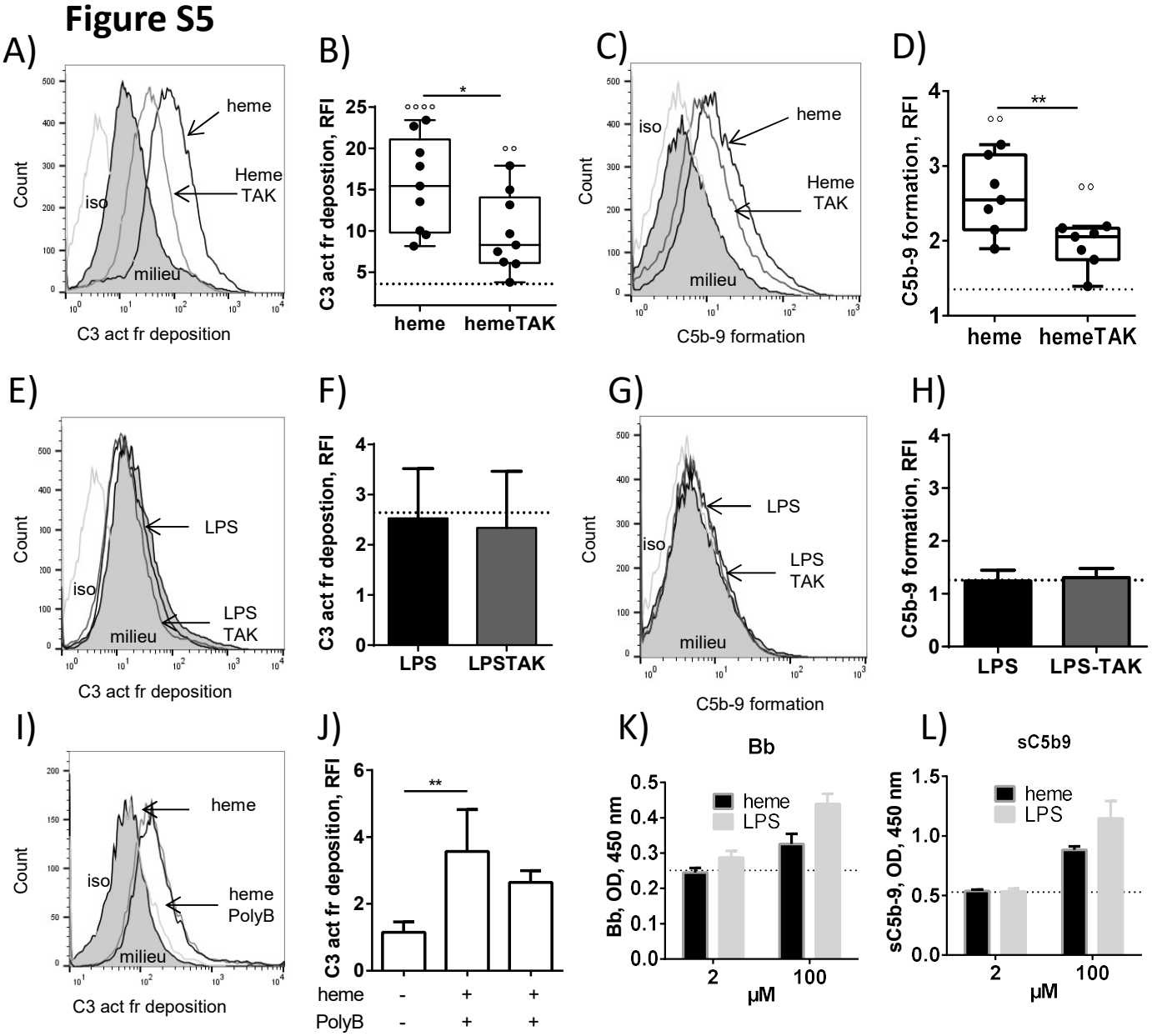


Supplementary figure S3: Liver stress response: A) Frozen liver sections from WT, TLR4-/- and C3-/- mice were stained for NGAL (red) (n≥3). B) mRNA levels in WT mice treated with heme, with or without Hx (n≥4). C) Staining of liver sections for NGAL (red) and CD31 (green) of mice, pre-treated with PBS, Irrelevant IgG (Irr IgG) or C5 blocking antibody BB5.1 (α-C5), followed by PHZ administration. D) mRNA levels in WT mice treated with PHZ in combination with irrelevant IgG or anti-C5 blocking antibody BB5.1 (α-C5). E) Staining of liver sections for C3b/iC3b (red) and CD31 (green) of mice, pre-treated with PBS, Irrelevant IgG (Irr IgG) or C5 blocking antibody BB5.1 (α-C5), followed by PHZ administration. C,E) The colocalization appears in orange. *: $p<0.05$; **: $p<0.005$, ***: $p<0.001$, Two-way ANOVA with Tukey's test for multiple comparisons. Values are mean +/- SEM.

Figure S4

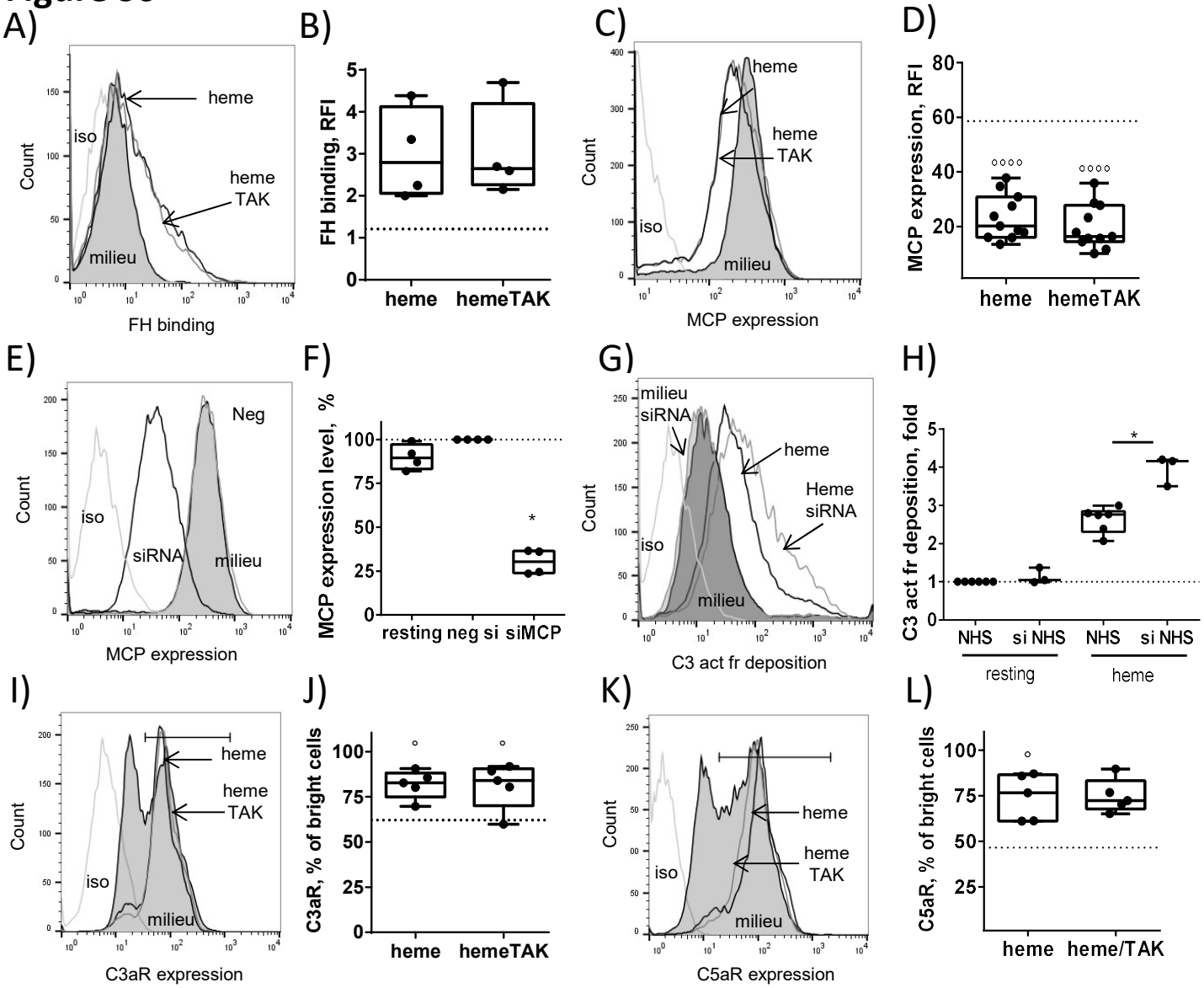


Supplementary Figure S4: Heme induces TLR4 signaling in a different manner than LPS. HUVEC were treated with 100µM of heme or with 200 ng/ml of LPS; with or without 400 nM of TAK-242 for 30 min at 37 °C. Cells were detached and stained for TLR4 ($n \geq 4$) (A,B,C,D) and CD14 (n=14) (E,F,G,H) by flow cytometry. I,J) Cells were incubated for 30 min in presence of 100 µM of heme. Phosphorylation of p38 (I) and NFkB (J) was stained and studied by immunofluorescence (green). Nuclei were stained with DAPI (blue). K) HUVEC were treated with 25 µM of heme are recovered after 30 min, 1 h, 2 h, 4 h and 6 h. After centrifugation, phosphorylation p65 was studied by WB of nuclear fraction. °°°: $p<0.001$; °°°°: $p<0.0001$; **: $p<0.005$. One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality. Values are box plots with median and Min/Max points in (B,F) and mean +/- SEM in (D,H).



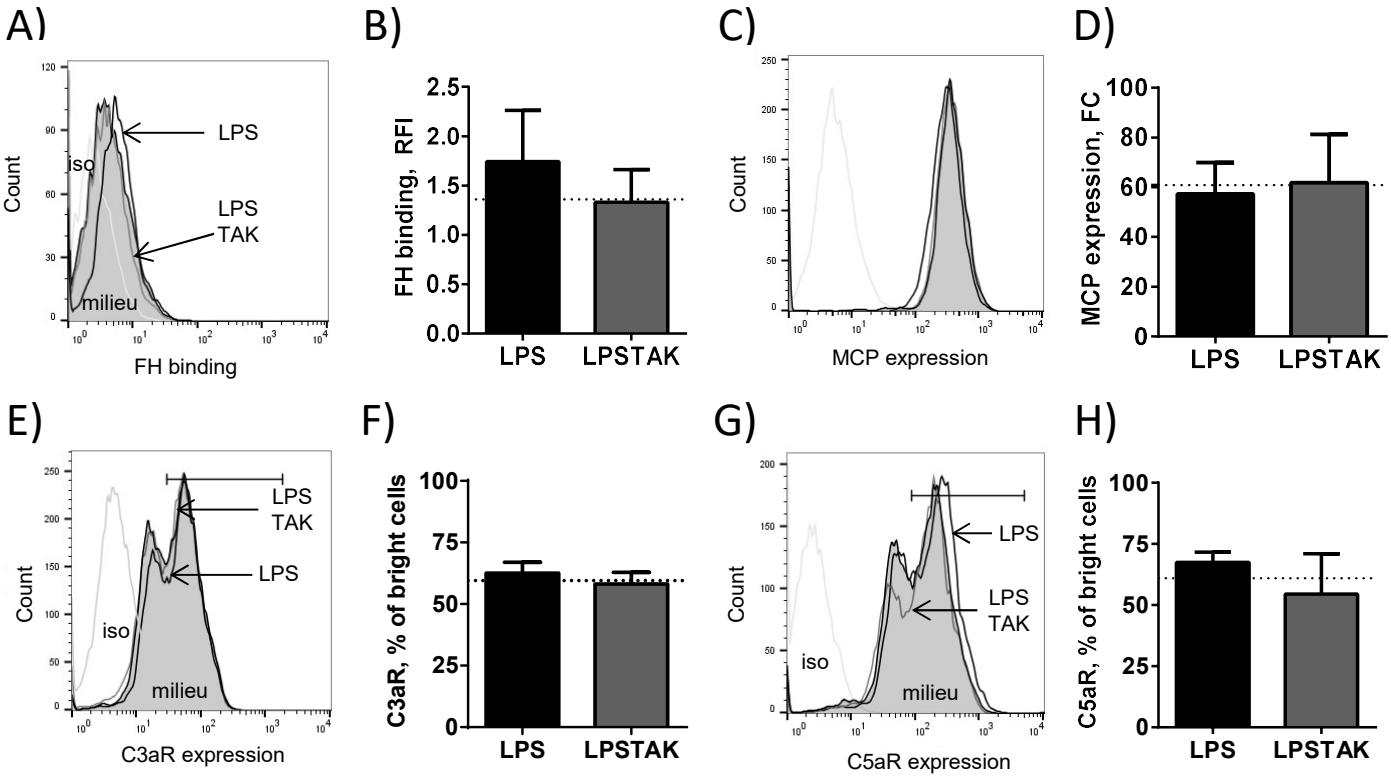
Supplementary Figure S5: Heme, but not LPS, mediates complement activation on EC is partially dependent on TLR4. HUVEC were treated with 100 µM of heme with or without 400 nM of TAK-242 for 30 min at 37 °C and studied by flow cytometry. A-D) After removing the supernatant, HUVEC were exposed to 33% NHS for 30 min at 37 °C. Cells were detached and stained for C3 activation fragments (C3 act fr) deposition (A,B), or C5b-9 formation (C,D). E-H) HUVEC were treated with 200 ng/ml of LPS with or without 400 nM of TAK-242 for 30 min at 37 °C. After removing the supernatant, HUVEC were exposed to 33% NHS for 30 min at 37 °C. Cells were detached and stained for C3 activation fragments deposition (E,F), or C5b-9 formation (G,H) by flow cytometry. I,J) HUVEC were treated with 200 ng/ml of LPS with or without 1 ug/mL of polyB for 30 min at 37 °C. After removing the supernatant, HUVEC were exposed to 33% NHS for 30 min at 37 °C. Cells were detached and stained for C3 activation fragments deposition. K,L) NHS was exposed to different doses of LPS or heme for 1h at 37°C. Released K) Bb and L) sC5b-9 were measured by ELISA. The dotted line represents the basal levels of C3 activation fragments (B,F) and C5b-9 (D,H) of Bb or sC5b-9 in the serum in K) and L), without addition of complement activator. °°: $p<0.005$; °°°°: $p<0.0001$; *: $p<0.05$; **: $p<0.005$. One Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality. Values are box plots with median and Min/Max points in (B,D) and mean +/- SEM in (F,H).

Figure S6



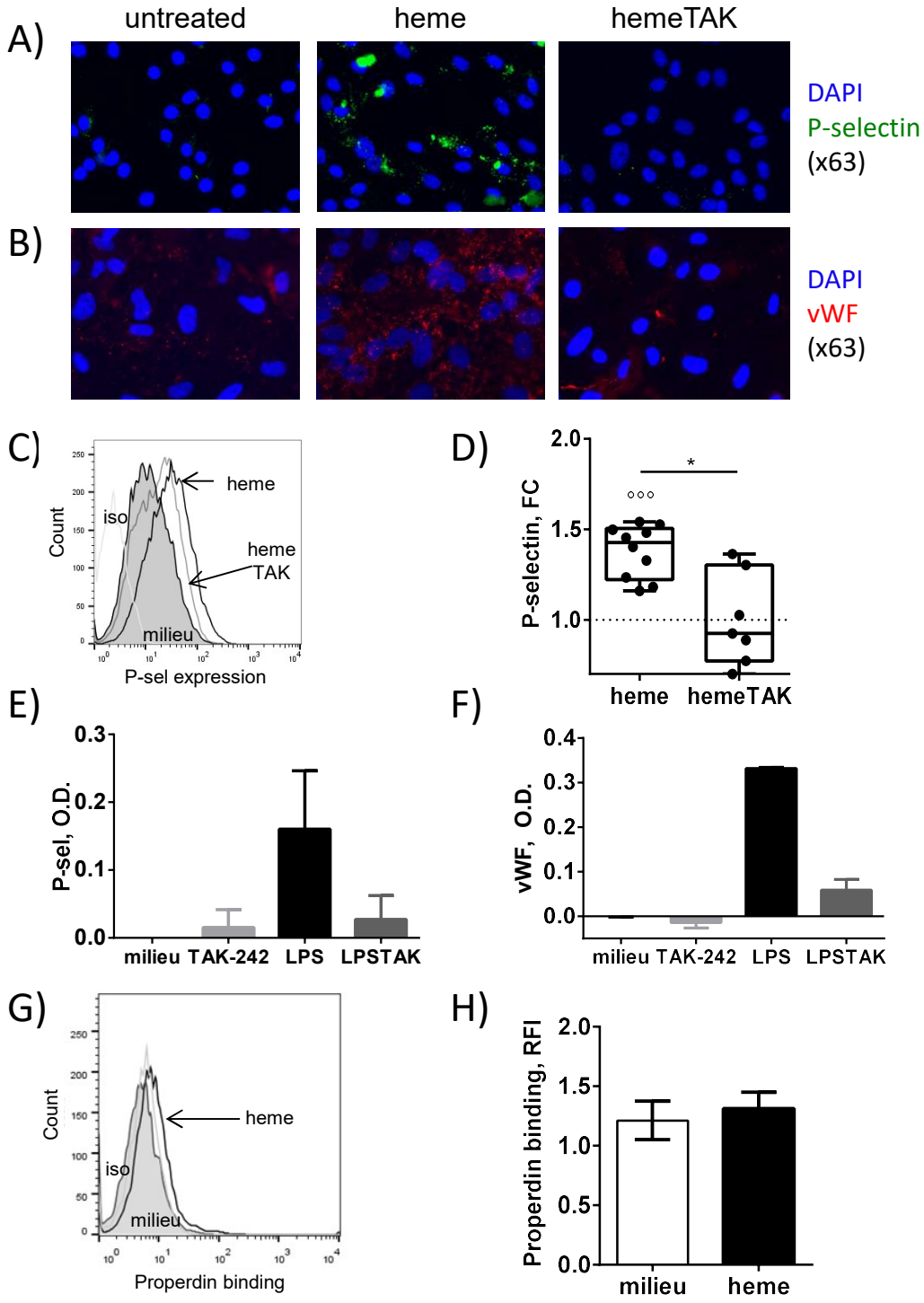
Supplementary Figure S6: Heme alters expression of complement related proteins on EC surfaces in TLR4-independent manner. A,B) After removing the supernatant, HUVEC were exposed to 33% NHS for 30 min at 37 °C. Cells were detached and stained for FH (n=5). C,D) Cells were detached and stained for MCP (n=13). E,F) Silencing of MCP in HUVEC reduced about 75% of MCP expression compare to basal level. G,H) HUVEC and MCP-silenced HUVEC were treated with 100 µM of heme for 30 min at 37 °C. After removing the supernatant, HUVEC were exposed to 33% NHS for 30 min at 37 °C. Cells were detached and stained for C3 activation fragments (C3 act fr) deposition (n=3). I-L) HUVEC were treated with 100 µM of heme with or without 400 nM of TAK-242 for 30 min at 37 °C. Cells were detached and stained for C3aR (n=5) (I,J) or C5aR (n=5) (K,L). °: $p<0.05$, °°°°: $p<0.0001$, Mann-Withney t-test compared to basal level, represented as dotted line. *: $p<0.05$, One-Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality. Values are box plots with median and Min/Max points.

Figure S7



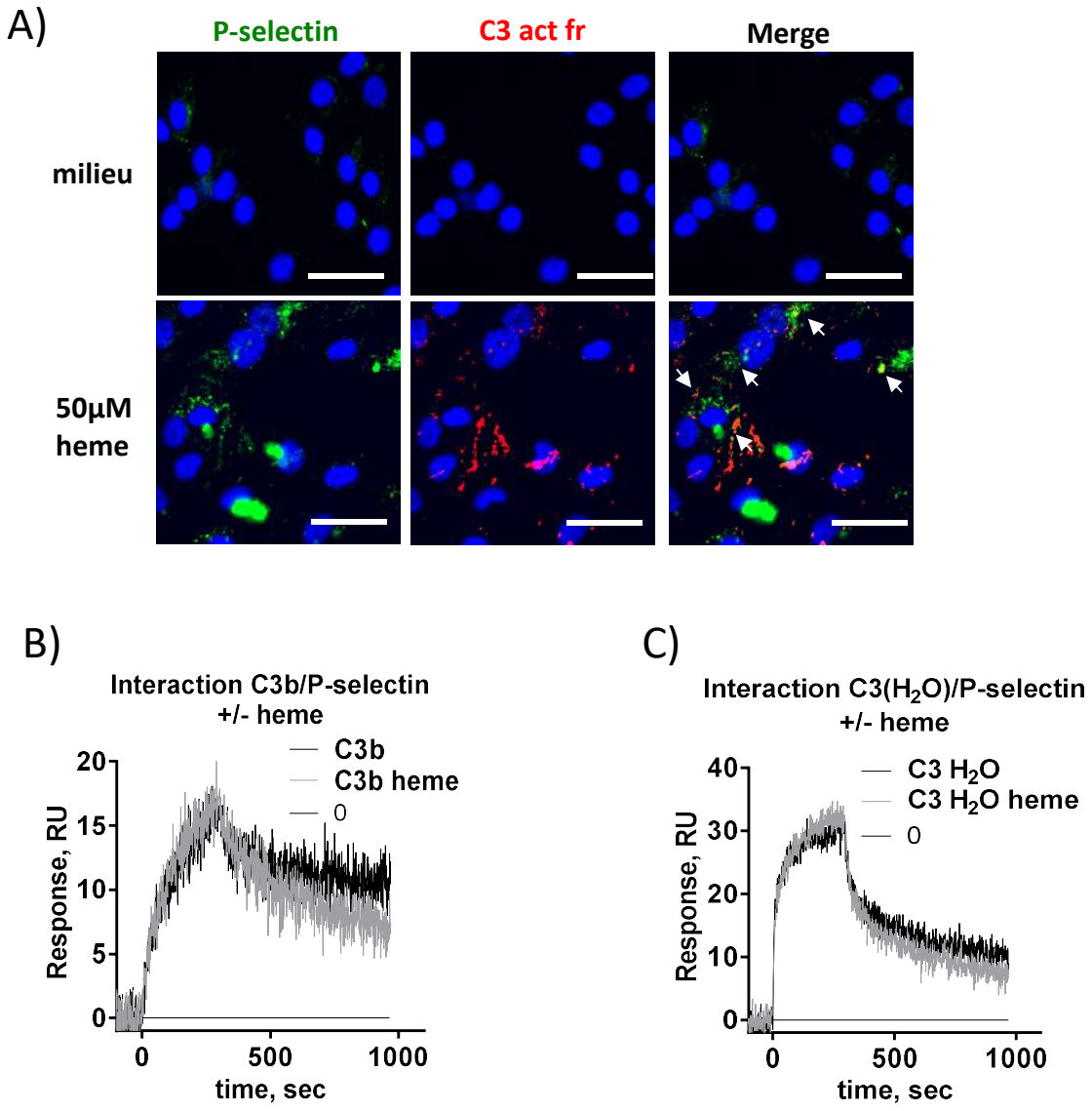
Supplementary figure S7: LPS does not alter expression of complement related proteins on EC surfaces. HUVEC were treated with 200 ng/ml of LPS with or without 400 nM of TAK-242 for 30 min at 37 °C. After removing the supernatant, HUVEC were exposed to 33% NHS for 30 min at 37 °C. Cells were detached and stained for FH (n=3) (A,B). HUVEC were treated with 200 ng/ml of LPS with or without 400 nM of TAK-242 for 30 min at 37 °C. Cells were detached and stained for MCP (C,D), C3aR (n=3) (E,F) of C5aR (n=3) (G,H) by flow cytometry. Values are mean +/- SD.

Figure S8



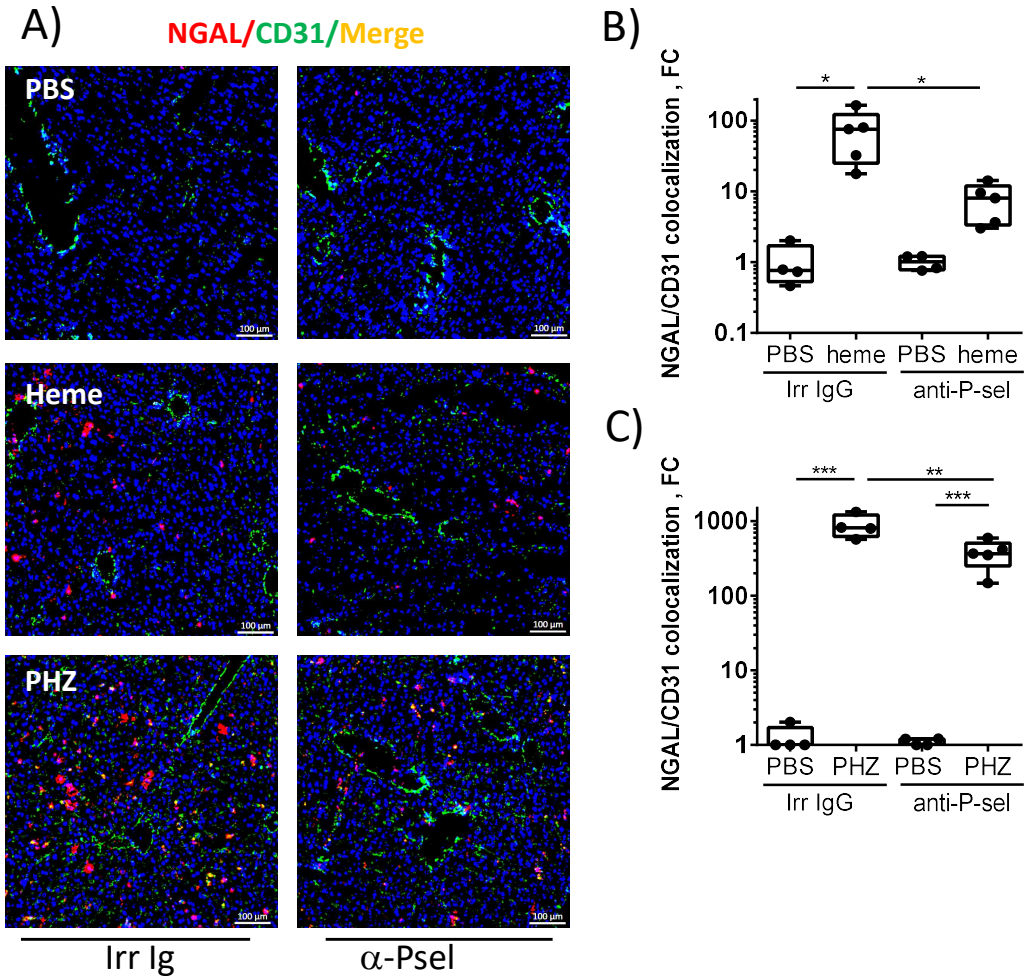
Supplementary figure S8: Both heme and LPS induce expression of P-selectin and release of vWF. HUVEC were treated with 100 µM of heme +/- 400 nM of TAK-242 for 30 min at 37 °C. HUVEC were treated with 100µM of heme with or without 400nM of TAK-242 for 30 mins at 37°C. Cells were stained for P-selectin (false color green) (A) or vWF (false color red) (B) by IF; magnification x63. Nuclei were stained with DAPI (blue). C,D) Cells were detached and stained for P-selectin (n=9, flow cytometry). HUVEC were treated with 200 ng/ml of LPS for 1 hour at 37 °C. Exocytosis of P-selectin (n=3) (E) and vWF (n=3) (F) were measured by cellular ELISA. G,H) HUVEC were treated with 100µM of heme or milieu for 30 min at 37 °C. After removing supernatant, HUVEC were exposed to 33% NHS, detached and stained for properdin and studied by flow cytometry. °°°: $p<0.001$, Mann-Withney t-test compared to basal level, represented as dotted line. *: $p<0.05$, One-Way ANOVA with Holm-Sidak's for multiple comparisons after Shapiro-Wilk test for normality. Values are box plots with median and Min/Max points (D), or mean +/- SD (E,F,H).

Figure S9



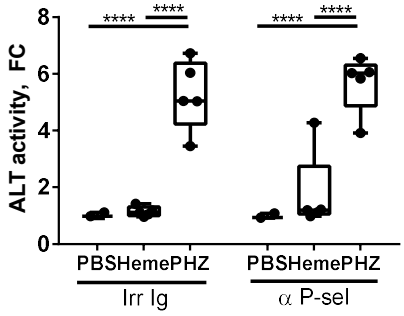
Supplementary figure S9: A) Colocalization of P-selectin (false color green) and C3-activation fragments (false color red) on heme-exposed HUVEC. Nuclei were stained with DAPI (blue)**.** Untreated cells are indicated as milieu. B,C) Presence of heme does not modify binding of C3b and C3(H_2O) on P-selectin. Interaction between C3b/P-selectin and C3(H_2O)/P-selectin in presence of 100 µM of heme were studied by surface plasmon resonance by injecting increased concentration of C3b/heme (B) or freeze/thaw native C3/heme (C) on P-selectin-coated chip.

Figure S10



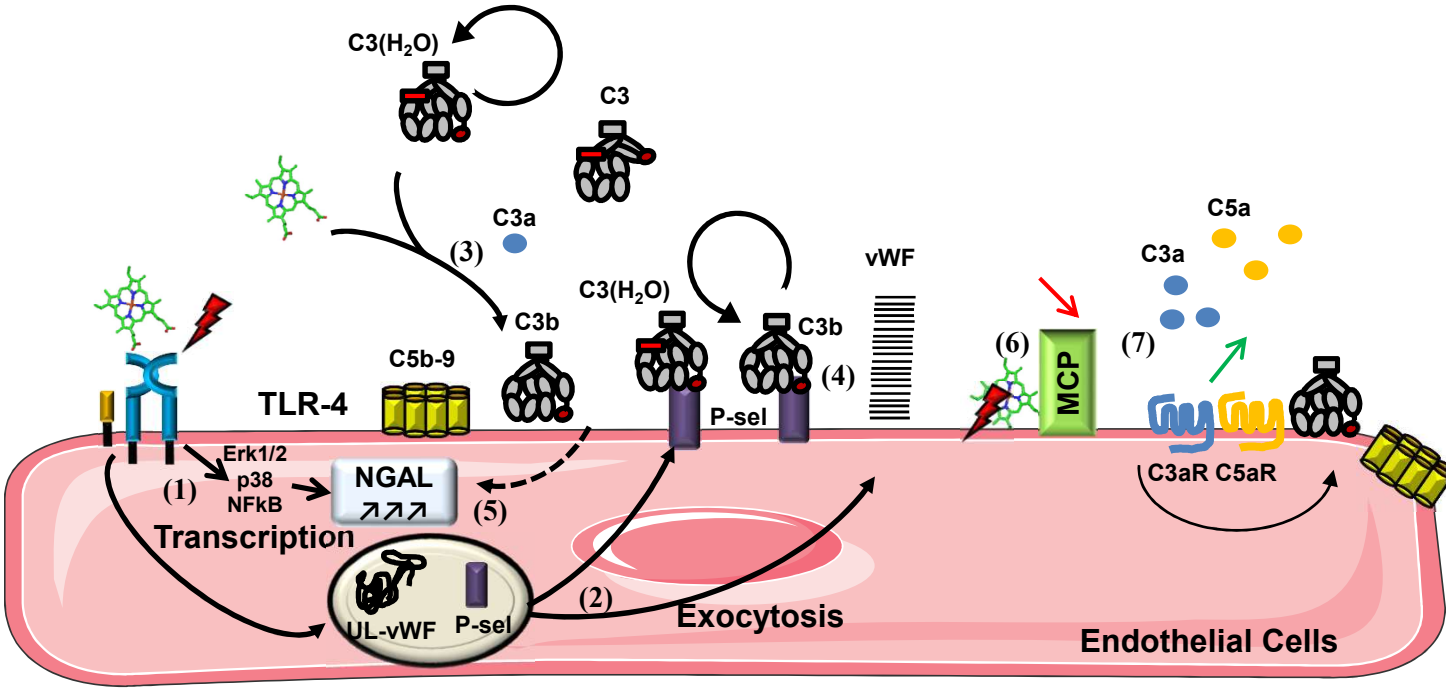
Supplementary figure S10: Anti-P-selectin administration prevents tissue stress response and complement activation in hemolytic conditions in vivo. WT mice were injected with PBS, heme or PHZ after first administration of an irrevelant antibody (Irr Ig) or blocking antibody against P-selectin (α P-sel). A) Examples of staining of double staining NGAL (false color red) and CD31 (false color green) in liver frozen sections by IF after PBS, heme and PHZ injection. The merge indicating co-localization is in orange. B-C) Quantification of the staining for NGAL in liver vessels in mice, treated with heme (B) or PHZ (C). *: p<0,05, **: p<0.005, ***: p<0.001. Two-Way ANOVA with with Tukey's test for multiple comparisons for (B-C). Values are box plots with median and Min/Max points (B,C).

Figure S11



Supplementary figure S11: ALT activity, measured by colorimetric. WT mice were injected with PBS, heme or PHZ after first administration of an irrevelant antibody (Irr Ig) or blocking antibody against P-selectin (αP-sel). ****: $p<0.0001$, Two-Way ANOVA with Tukey's test for multiple comparisons. Values are box plots with median and Min/Max points.

Figure S12



Supplementary figure S12: Schematic view of complement activation mechanism on EC during hemolysis. (1) Heme, released from hemolysis, induces TLR4 signaling on endothelial cells, measured by Erk1/2, p38 and NFkB pathways transduction as well as (2) a rapid exocytosis of Weibel Palade bodies (WPB), leading to the von Willebrand factor (vWF) release and the P-selectin (P-sel) expression. (3) In parallel, heme induces C3(H_2O) generation and promotes the formation of alternative C3 convertases in fluid phase, which cleave native C3 from serum. (4) C3(H_2O) and C3b are anchored on cell membrane, allowing local formation of C3 convertases, release of anaphylatoxins C3a and C5a, till C5b-9 formation. (5) TLR4 signaling and complement activation induce endothelial injury and expression of stress response genes, such as NGAL. In addition, on human endothelial cells, (6) decrease of MCP and (7) upregulation of C3aR and C5aR expressions also promote cell stress and complement deposits. Blockade of each of these steps (heme, TLR4, P-selectin, C3 and C5) could attenuate endothelial and tissue injury.